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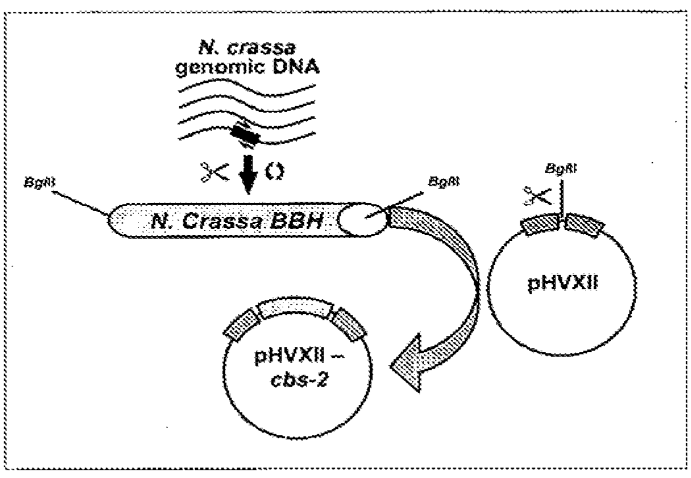
(54) Title: METHOD OF PRODUCING A CARNITINE-SYNTHESISING MICRO-ORGANISM



(57) Abstract: The invention discloses a method of producing a micro-organism that can biosynthesise carnitine from a non-carnitine synthesising micro-organism, the method including the step of transforming the non-carnitine synthesising micro-organism with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH). The transformed micro-organism is capable of producing carnitine when cultured in the presence of gamma-butyrobetaine. A method of identifying a carnitine-producing micro-organism is also disclosed, the method including the steps of applying a micro-organism to a synthetic agar medium which does not contain carnitine and is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain; culturing the micro-organism; and detecting the presence of a zone in the agar medium formed by carnitine-producing micro-organisms. The transformed micro-organism may be used to enhance the nutritional value of foods and beverages, such as bread and beer.

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METHOD OF PRODUCING A CARNITINE-SYNTHESISING MICRO-ORGANISM

BACKGROUND OF THE INVENTION

The invention relates to a method for producing a micro-organism that is capable of synthesising carnitine, and also describes a method of identifying whether a micro-organism is capable of synthesising carnitine.

L-Carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) is a quaternary ammonium compound that was first discovered in muscle extracts in 1905 (Bremer, 1983). In 1952, it was shown that the mealworm *Tenebrio molitor* is dependent on carnitine for survival, generating new interest in this molecule, which was named vitamin B_T (Carter et al., 1952). Later investigations showed that most eukaryotic organisms could synthesise L-carnitine from trimethyllysine as a precursor (Vaz and Wanders, 2002). Nevertheless, carnitine deficiencies occur and they are debilitating diseases, frequently due to genetic mutations (Bonnefont et al., 1999; Lahjouji et al., 2001). Such diseases are characterised by low levels of carnitine in either the serum or in specific tissues. In most cases, patients respond favourably to exogenous dietary supplementation of carnitine (Pons and De Vivo, 1995). In recent times, L-carnitine has also been used for symptomatic treatment in cases of diseases, such as chronic fatigue syndrome, coronary vascular disease, hypoglycemia and muscular myopathies (Kelly, 1998). In addition, carnitine is widely used in nutritional products, such as energy drinks, weight loss supplements and baby formulae (Carter et al., 1995).

In mammalian cells, carnitine is an essential component of the mitochondrial carnitine cycle that is responsible for the transfer of activated long-chain fatty acids into the mitochondria or peroxisome for β-oxidation (Bieber, 1988). In the yeast *Saccharomyces cerevisiae*, on the other hand, β-oxidation occurs solely in the peroxisomes (Kunau et al., 1988). Van Roermund et al. (1995) showed that exogenous carnitine was essential for growth on fatty acids as sole carbon source in

the absence of the glyoxylate cycle citrate synthase, Cit2p. Later, Swiegers et al. (2001) showed that in the Δ*cit2* strain, carnitine is essential for growth on all non-fermentable carbon sources. Therefore, *S. cerevisiae* is unable to biosynthesise carnitine endogenously but relies on exogenous carnitine, which is transported into the cell by the general amino acid membrane transporter Agp2p (van Roermund et al., 1999; Swiegers et al., 2001).

Mammals, plants and some fungi are able to biosynthesise carnitine from ε-*N*-trimethyllysine (TML) (Lindstedt and Lindstedt, 1970; Kaufman and Broquist, 1977; Bremer, 1983). In mammals, TML is provided by the lysosomal hydrolysis of proteins that contain this amino acid as a result of the post-translational modification of lysine residues (Bremer, 1983). However, in *Neurospora crassa*, free lysine is trimethylated in the cytosol (Borum and Broquist, 1977). In the first step of carnitine biosynthesis, TML is hydroxylated to β-hydroxy-ε-*N*-trimethyllysine by ε-*N*-trimethyllysine hydroxylase (TMLH; EC1.14.11.8) (Rebouche and Engel, 1980; Bremer, 1983). Subsequently, β-hydroxy-ε-*N*-trimethyllysine is cleaved into γ-trimethylamino-butyraldehyde and glycine by β-hydroxy-ε-*N*-trimethyllysine aldolase (Rebouche and Engel, 1980; Bremer, 1983). The aldehyde is then oxidised by γ-trimethylaminobutyraldehyde dehydrogenase to form γ-butyrobetaine (Hulse and Henderson, 1980; Rebouche and Engel, 1980; Bremer, 1983). Finally, γ-butyrobetaine is hydroxylated at the 3-position by γ-butyrobetaine hydroxylase to form L-carnitine (Figure 1) (BBH; EC 1.14.11.1) (Englard, 1979; Rebouche and Engel, 1980; Bremer, 1983).

The identity of some of the intermediate metabolites of the carnitine biosynthesis pathway was first elucidated in the filamentous fungus *Neurospora crassa*, using isotope-labelling experiments (Kaufman and Broquist, 1977). The genes encoding the enzymes required for the catalysis of three of the four reactions required for carnitine biosynthesis have been characterised at the molecular level, in rats and humans (Vaz et al., 1998; Galland et al., 1999, Vaz et al., 1999; Vaz et al., 2000; Vaz et al., 2001).

The applicant has therefore identified a need for producing a strain of *S. cerevisiae* that can produce carnitine. The applicant has also identified a need for a simple and inexpensive method of determining whether or not a micro-organism is capable of producing carnitine.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided a method of producing a micro-organism that can biosynthesise carnitine from a non-carnitine synthesising micro-organism, the method including the step of:

transforming the non-carnitine synthesising micro-organism with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH).

The micro-organism may be a yeast strain, such as *Saccharomyces cerevisiae.*

The transformed strain may be cultured in the presence of γ-butyrobetaine in order for the strain to produce carnitine.

The γ-butyrobetaine hydroxylase may be a *Neurospora crassa* γ-butyrobetaine hydroxylase, and the nucleotide sequence may be a genomic fragment having the nucleotide sequence as set out in SEQ ID NO: 1 (Figure 7).

The *S. cerevisiae* strain may be a FY23 strain.

The non-carnitine synthesising micro-organism may be identified by:

applying the micro-organism to a synthetic culture medium containing a non-fermentable carbon source and no carnitine, and which is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain;

culturing the micro-organism; and

detecting formation of a zone produced in the culture medium in the region of the micro-organism if it is a carnitine-producing micro-organism, or detecting no such zone if the micro-organism is not able to produce carnitine.

The above identification steps may also be performed to determine whether the method of producing a micro-organism that can biosynthesise carnitine has been successful.

According to a second embodiment of the invention there is provided a strain of *Saccharomyces cerevisiae* that can produce carnitine in the presence of γ-

butyrobetaine, which has been transformed with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH) substantially as described above.

According to a third embodiment of the invention, there is provided a method of identifying a carnitine-producing micro-organism, the method including the steps of:

applying a micro-organism to a synthetic culture medium containing a non-fermentable carbon source, no carnitine and coated with a layer of *Saccharomyces cerevisiae Δcit2* strain;

culturing the micro-organism; and

detecting formation of a zone produced in the culture medium in the region of the micro-organism if the micro-organism is a carnitine-producing micro-organism.

The culture medium may be illuminated or transilluminated in order to detect formation of the zone.

The culture medium may be agar, and the non-fermentable carbon source may be ethanol. For example, the culture medium may be 2% ethanol synthetic agar medium.

The micro-organism may be cultured for a period of about 10 days at about 30 °C.

According to a fourth embodiment of the invention, there is provided a method of producing carnitine, the method including the step of culturing in the presence of γ-butyrobetaine hydroxylase (BBH) a micro-organism that has been transformed as above.

The micro-organism may be included in the production of a beverage or food product, such as beer or bread.

According to a fifth embodiment of the invention, there is provided a method of enhancing the nutritional value of a beverage or food product, the method including the step of incorporating a microorganism that has been transformed as described above in the process of manufacturing the food or beverage product.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a reaction scheme for hydroxylation of γ-butyrobetaine to L-carnitine by γ-butyrobetaine hydroxylase;

Figure 2 shows the alignment of the 671 amino acids expressed *N. crassa* BBH homologue (SEQ ID NO: 2) to human, *Caenorhabditis elegans* and *Pseudomanos* BBH proteins;

Figure 3 shows a carnitine large-scale plate screen. (A) Strains were grown on glucose synthetic medium and streaked on a 2% ethanol synthetic medium agar plate with 10 mg/l γ-butyrobetaine and with a thin mat of Δ*cit2* cells grown on synthetic glucose medium, which were washed twice with sterile distilled water before plating. Cells were grown for 10 days at 30°C. The production of L-carnitine by the strain expressing a functional BBH results in the secretion of carnitine, which complements the surrounding Δ*cit2* strains and resulted in the formation of a zone. (B) Carnitine secretion plate assay for identification of endogenous biosynthesis and secretion of carnitine. Yeasts were grown on glucose synthetic medium before they were streaked onto a 2% ethanol synthetic medium agar plate with a thin mat Δ*cit2* cells. Cells were grown for 10 days at 30°C. Yeast strains with endogenous L-carnitine biosynthesis and secretion could be identified by the zone formation (D5, D4 and D2). (D5) *Yarrowia lipolytica*; (D4) *Rhodotorula graminis*; (D2) *Candida curvata*. The controls were the *S. cerevisiae* laboratory strain FY23 (A1) and industrial commercial wine strain VIN13 (A2). Other non-zone forming yeast represents a various collection of different genus and species. To improve visual detection of zones, plates were transilluminated with light and photos taken. Biosynthesis of carnitine in zone forming strains was verified using electrospray mass spectrometry as described below;

Figure 4 shows (A) photographs of petri dishes on which strains were grown for 4 days at 30°C on synthetic glycerol (3%) medium and synthetic glycerol medium with 10 mg/l γ-butyrobetaine; and (B) growth curves of strains

and transformants: FY23 wild type strain (▲); FY23Δ*cit2* strain (△); FY23 wild type strain with *cbs-2* (■); FY23Δ*cit2* strain with *cbs-2* (□). Each strain was grown in 100 ml of synthetic glycerol (3%) medium plus 10 mg/l γ-butyrobetaine at 30°C;

Figure 5 shows the measurement of intracellular carnitine and acetylcarnitine using electrospray mass spectrometry. (A) FY23 wild type strain with *cbs-2* and (B) FY23 wild type strain was grown on synthetic glycerol (3%) medium with 10 mg/l γ-butyrobetaine for 4 days at 30°C, after which cells were harvested and intracellular carnitine and acetylcarnitine levels were determined. Carnitine has a parent ion of 162 and the daughter fragment of 43 was measured. Acetylcarnitine has a parent ion of 204 and a daughter ion of 85 was measured. The level of intracellular carnitine measured for *cbs-2* transformed cells were 897 ng/gWW and acetylcarnitine 1151 ng/gWW;

Figure 6 shows a diagrammatic representation of the cloning strategy employed in the construction of the *N. crassa* BBH containing construct pHVXII-*cbs-2*. The symbol "✂" indicates the use of restriction enzymes, and "" refer to the use of the polymerase chain reaction;

Figure 7 shows the nucleotide sequence of a 2016bp fragment (SEQ ID NO: 1), encoding a putative protein with BBH homoloy, cloned from *N. crassa* genomic DNA in FASTA format; and

Figure 8 shows the putative amino acid sequence (SEQ ID NO: 2) encoded by the pHVXII-*cbs-2* construct, which includes the entire area of BBH homology, in FASTA format.

DETAILED DESCRIPTION OF THE INVENTION

A method of producing a micro-organism that can biosynthesise carnitine from a non-carnitine synthesising micro-organism is described herein. The method includes the

step of transforming the non-carnitine synthesising micro-organism with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH).

The micro-organism is generally a yeast strain, such as *Saccharomyces cerevisiae*, and more particularly a FY23 strain, although it may be any other type of micro-organism that is able to transport butyrobetaine into the cell, as the transformed strain is cultured in the presence of γ-butyrobetaine in order for the strain to produce carnitine.

The γ-butyrobetaine hydroxylase is generally a *Neurospora crassa* γ-butyrobetaine hydroxylase, and the nucleotide sequence thereof may be a genomic fragment having the nucleotide sequence as set out in SEQ ID NO: 1 (Figure 7).

Also disclosed is a method of identifying a carnitine-producing micro-organism, by applying a micro-organism to a synthetic culture medium which contains a non-fermentable carbon source but no carnitine, and which is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain. A typical culture medium is agar, and a suitable non-fermentable carbon source is ethanol. For example, the culture medium may be 2% ethanol synthetic agar medium. The micro-organism is then cultured, typically for a period of about 10 days at about 30 °C. The formation of a zone produced in the culture medium in the region of the micro-organism will be detectable, such as by illumination or transillumination, if the micro-organism is a carnitine-producing micro-organism. No such zone will be detected if the micro-organism is not a carnitine-producing micro-organism.

L-Carnitine is a quaternary ammonium compound that plays an essential role in the transfer of activated acyl-residues across intra-cellular membranes. Most eukaryotes can neo-synthesise carnitine, but recent data show that this is not the case in the yeast *Saccharomyces cerevisiae*. The filamentous fungus *Neurospora crassa* was one of the first organisms used to identify the precursor and intermediates of the carnitine biosynthesis pathway. In this organism, the precursor trimethyllysine is converted in a four-step process to carnitine. In the last step of this pathway, γ-butyrobetaine is hydroxylated to form carnitine in a reaction catalysed by γ-butyrobetaine hydroxylase (BBH).

A novel plate screen that can be used to identify genomic fragments of *N. crassa* that functionally express BBH is described herein. Using this plate screen, a genomic fragment encoding the *N. crassa* γ-butyrobetaine hydroxylase (BBH) was identified and the gene designated *cbs-2*. The invention teaches that a wild type yeast strain transformed with the *cbs-2* gene can use exogenous γ-butyrobetaine to produce carnitine, and expression of this gene is able to rescue the growth defect of a *Δcit2* strain on non-fermentable carbon sources, without carnitine, in the presence of γ-butyrobetaine.

The invention will now be described in more detail by way of the following non-limiting examples.

Examples:

Yeast strains and plasmids

FY23 (*MATa leu2 trp1 ura3*) was used as a wild-type strain (Winston et al., 1995). The FY23Δ*cit2* (*MATa leu2 ura3 cit2::TRP1*) was used as the glyoxylate citrate synthase deficient strain (Swiegers et al., 2001). A 2016 bp fragment (Figure 7; SEQ ID NO: 1) was cloned from *N. crassa* genomic DNA using the primers NcBBH-F (5'-GATCAGATCT **ATG** AAA GTC GAC AAG GAA GCC GGC AA-3') (SEQ ID NO: 3) and NcBBH-R (5'-GATCAGATCT **TTA** TGC GTT CCA GTT CAC CGT GCC CAA-3') (SEQ ID NO: 4) with introduced restriction sites. Genomic DNA was extracted from strain PPRI 3338 (National Collection of Fungi, Agricultural Research Council, Pretoria, South Africa). The fragment was cloned into expression vector pHVXII into the *Bgl*II site under the regulation of the *PGK1* promoter (Volschenck et al., 1997; represented in Figure 6). Sequencing was done using the ABI-Prism sequencer. The *S. cerevisiae* gene *YHL021c* was amplified by PCR from genomic DNA from strain FY23 using the primers YHL-F (5'-GATCGAATTC **ATG** CTA AGA TCA AAT TTA TGC AGA GGA-3') (SEQ ID NO: 5) and YHL-R (5'-GATCCTCGAG **TTA** TTT GTA CTG AGG AAA CTT CTC TTC-3') (SEQ ID NO: 6) with introduced restriction sites. The fragment was cloned into expression vector pHVXII into the *Bgl*II site under the yeast *PGK1* promoter. Constructs were transformed into the yeast strains using the lithium acetate procedure (Becker and Gaurente, 1991).

Media and growth conditions

Escherichia coli carrying plasmids were grown in Luria Bertani (LB) broth with 10 mg/l ampicillin. Yeast strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose), synthetic glucose medium (6.7 g/l yeast nitrogen base without amino acids, 2% glucose, amino acids as required), and synthetic glycerol medium (6.7 g/l yeast nitrogen base without amino acids, 3% glycerol, amino acids as required). Media was prepared using double distilled water.

Intra-cellular carnitine extraction

Transformants were grown on synthetic glucose medium for two days and then inoculated in 100 ml synthetic glycerol medium with 10 mg/l γ-butyrobetaine and grown for 4 days at 30°C. Cells were harvested by centrifuging 5 min at 5000 rpm and washed with 40 ml double distilled water and harvested again using the same procedure. Cells were resuspended in 1 ml double distilled water, transferred to a 1.5 ml microcentrifuge tube and harvested at 12 000 rpm for 2 min. Wet weight was determined by weighing the cells and the microcentrifuge tube after all the supernatant was removed by pippeting. The cells were resuspended in 0.2 ml double distilled water. The cells were disrupted by adding 0.16 g glass beads and vortexed for 30 min at 8°C. The cells were then vortexed for 10 min at 12 000 rpm and 0.1 ml of the supernatant added to 0.9 ml acetonitrile and stored at -20°C. Before ES-MS analysis, the solution was centrifuged for 10 min at 12 000 rpm to remove all protein precipitates and the supernatant used for analysis.

HPLC-electrospray mass spectrometry

Mass spectrometry was performed on a Micromass (Manchester, UK) Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. Solvent A (acetonitrile/water/formic acid: 30/70/0.05 (v/v/v) was used as a carrier solvent and was supplied to the ionisation source by a LKB/Pharmacia (Sweden) pump. For direct injection of the carnitine and acetylcarnitine standards, the flow rate was 20 µl/min and 5 µl of the standard was injected through a Rheodyne injection valve. The molecular ion ([M+H]+) of carnitine and acetylcarnitine was observed using a capillary voltage of 3.5 kV, source temperature of 80°C and a cone voltage setting of 20 V. To obtain the fragment pattern of carnitine and acetylcarnitine, the molecular ion was dissociated in the fragmentation cell by collision-induced dissociation at an argon pressure of 2.8×10^{-3} mbar (2.8×10^{2} Pa) applying collision energy of 20 eV. The resultant fragments

were scanned in the second analyser. Quantitation of carnitine and acetylcarnitine in the incubation samples was accomplished by LCMSMS. A Luna C18 150x2 mm (3μ) column was used for separation, with solvent A as the mobile phase at a flow rate of 100 μl/min delivered by the above mentioned pump. Five microliter of sample was injected by a Waters 747 autosampler. The eluent from the column was directed into the electrospray ionisation source of the mass spectrometer. The capillary voltage, cone voltage, argon pressure and collision energy were as mentioned above. Detection was by multiple reaction monitoring, using the molecular ions of carnitine and acetylcarnitine as precursor ions and the fragments at m/z = 43 and m/z = 85 as product ions, respectively. Chromatographic peaks representing carnitine and acetylcarnitine were integrated and the concentration in the incubation samples were calculated from a dilution range of known concentrations of standard carnitine and acetylcarnitine in distilled water and diluted to a final concentration of 90/10 (v/v): acetonitrile/15 mM Tris.HCl. The calculations were automatically performed by the Quantify program of MassLynx and expressed as ng/ml.

Identification of a *N. crassa* BBH homologue

BBH protein sequences from different organisms are highly homologous to each other and to TMLH protein sequences. BBH and TMLH are part of a family of α-ketoglutarate-dependent, non-haem ferrous iron dioxygenases (Vaz and Wanders, 2002). However, when the BBH and TMLH proteins are compared to other proteins using BLAST searches (NCBI), reduced homology is found, indicating that these enzymes form a separate class of dioxygenases.

Searching the *N. crassa* Genome Database (NCGD) resulted in the identification of 2 putative proteins with high homology to human, rat and mouse BBH protein sequences (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). The first corresponds to a TMLH previously cloned and identified in a laboratory at Stellenbosch University (Swiegers et al., 2002), whereas the second was a novel gene encoding a hypothetical protein (NCU06891.1). The BBH homologous gene predicted by the NCGD consists of 5 predicted exons totalling 3786 bp and translating a hypothetical protein of 1262 aa (http://www-genome.wi.mit.edu/annotation/fungi/neurospora, Feature Search: NCU06891.2; SEQ ID NOS: 8 and 9). This is in strong contrast to the other known BBH proteins of humans, rats, mice and *Pseudomonas*, the length of which varies between 340-380 aa. However, homology to BBH proteins is only found for the protein

sequence translated by the last exon (exon 5) as described in the feature map of the hypothetical protein on the NCGD. The other translated exons do not show homology to any known protein.

Using the *N. crassa* genomic DNA, a 2016 bp fragment (Figure 7, SEQ ID NO: 1) (encoding a putative protein of 671 aa which includes the entire area of BBH homology, Figure 8 (SEQ ID NO: 2)), was cloned into a yeast expression vector, pHVXII under regulation of the *PGK1* promotor. Sequencing confirmed that the correct genomic area was cloned. Homology of the 671 aa putative protein to other known BBH proteins from humans, mouse and *Pseudomonas* are shown in Figure 2. However, the 671 aa putative proteins contained a 111 aa N-terminal and 110 aa C-terminal flanking regions without any homology to known BBH proteins. The C-terminal domain contains a six-fold repeat of the sequence "PKVEE" (SEQ ID NO: 7). Some 'additional' internal sequences, which contained GGGG repeats, were also present within the BBH homologous area, similar to what was observed for the *N. crassa* TMLH where an 11 residue poly P region and an "AAAAA" are found within the TMLH homologous area (Swiegers et al., 2002).

Screening of carnitine producing transformants

A large-scale screen was developed to identify microorganisms producing carnitine. The screen is based on the carnitine-dependent Δ*cit2* strain, which, after thorough washing, is plated as a mat on a synthetic agar medium containing a non-fermentable carbon source (e.g. ethanol) and no carnitine. On such plates, growing colonies of microorganisms that biosynthesise carnitine produce a zone due to the complementation of the Δ*cit2* mutant by carnitine. In this way, various microorganisms that produce carnitine were identified, including the yeasts *Yarrowia lipolytica*, *Rhodotorula graminis* and *Candida curvata* (Figure 3B). Endogenous carnitine biosynthesis by these strains was verified through intracellular carnitine/acetylcarnitine measurements using a novel HPLC-electrospray mass spectrometry (ESMS) method that is described above.

By adapting this large-scale screen, *S. cerevisiae* strains encoding functional BBH genomic fragments could be observed. As in the previous case, washed Δ*cit2* cells were plated on a non-fermentable carbon source but in this instance, γ-butyrobetaine was added to the media to provide the necessary intermediate. Transformed wild type

strains forming zones would indicate functionally expressed BBH due to the formation of carnitine from γ-butyrobetaine and its subsequent secretion into the growth medium. Expressing the 2013 bp genomic fragment from the hypothetical protein NCU06891.1, resulted in the formation of a zone (Figure 3A). It was thus concluded that the gene encoded a BBH, and therefore the gene was named *cbs-2* for "carnitine biosynthesis gene no. 2".

Complementation of the carnitine-dependent Δ*cit2* strain by BBH

The pHVXII-*cbs-2* construct encoding the 671 aa BBH homologue was transformed into FY23Δ*cit2* in order to see if the transformed strains were able to grow on medium containing the precursor γ-butyrobetaine. The transformants were streaked on synthetic glycerol media with and without γ-butyrobetaine. Strain FY23Δ*cit2* transformed with the *cbs-2* construct, grew in the presence of γ-butyrobetaine, whereas the FY23Δ*cit2* transformed with the vector did not grow on any of the media tested (Figure 4A). Wild type strain FY23 grew normal on both glycerol media tested. The growth effect on glycerol agar plates were also clearly demonstrated on glycerol liquid media where the FY23Δ*cit2* strain transformed with the *cbs-2* construct grew almost like the wild type strain and the FY23Δ*cit2* strain transformed with the vector alone did not grow (Figure 4B). Interestingly, the FY23 wild type transformed with *cbs-2* grew slightly better than the FY23 wild type transformed with vector alone indicating that production of carnitine can be advantageous for the cell or that γ-butyrobetaine may be slightly toxic to the cell. These data suggest that the γ-butyrobetaine in the growth medium is taken up by the Δ*cit2* strain and converted to carnitine endogenously, which allows the carnitine shuttle to function and therefore promote the production of energy and subsequent growth. To confirm this conclusion, carnitine and acetylcarnitine measurements were done using ESMS. Intra-cellular carnitine measurements were made after wild type strains were grown on synthetic medium containing glycerol and synthetic medium containing glycerol with γ-butyrobetaine. No carnitine could be measured in FY23 wild type and FY23 transformed with *cbs-2* in synthetic glycerol medium. When γ-butyrobetaine was added, the FY23 transformed with *cbs-2* gene showed high amounts of carnitine and acetylcarnitine, indicating that carnitine was produced and the carnitine shuttle was active (Figure 5). No carnitine could be measured in the FY23 wild type strain transformed with the vector alone, in the presence of γ-butyrobetaine. A total of 897 ng/gWW of intracellular carnitine and 1151

ng/gWW intracellular acetylcarnitine was measured. *S. cerevisiae* only has carnitine acetyltransferase activity, so acetylcarnitine is the only carnitine ester that can be formed (Swiegers et al., 2001). Carnitine production of *cbs-2* transformed strains could also be confirmed in glucose containing medium.

Discussion

In this study, a *S. cerevisiae* strain was genetically engineered that could biosynthesise carnitine from γ-butyrobetaine. A *N. crassa* genomic fragment was cloned that expressed a functional BBH, which could biosynthesise carnitine from γ-butyrobetaine. In addition, the BBH could suppress the growth defect of the carnitine-dependent Δ*cit2* strain when cells were grown on glycerol synthetic medium containing γ-butyrobetaine.

The use of the large-scale selection screen could be useful to isolate carnitine overproducing mutants through monitoring the zone sizes. It can also be used to identify the novel carnitine biosynthesis genes from a variety of organisms. The use of carnitine producing strains of *S. cerevisiae* will increase the nutritional value of foods such as bread and beverages such as beer and wine. In addition, carnitine has recently been shown to protect *S. cerevisiae* from stress conditions (Lee et al., 2002). This would be an additional advantage to *S. cerevisiae* strains.

It will be apparent to those skilled in the art that other non-carnitine synthesising microorganisms, when transformed with this gene, would be able to synthesise carnitine from butyrobetaine, unless unable to transport this compound into the cell. This invention is therefore not intended to be limited to strains of *Saccharomyces cerevisiae*. Various alterations, modifications and other changes may also be made to the invention without departing from the spirit and scope of the present invention. It is therefore intended that the claims cover or encompass all such modifications, alterations and/or changes.

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CLAIMS:

1. A method of producing a micro-organism that can biosynthesise carnitine, the method including the step of:

transforming a non-carnitine synthesising micro-organism with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH).

2. A method according to claim 1, wherein the micro-organism is a yeast strain.

3. A method according to claim 2, wherein the yeast strain is *Saccharomyces cerevisiae*.

4. A method according to any one of claims 1 to 3, wherein the transformed strain is cultured in the presence of γ-butyrobetaine in order for the strain to produce carnitine.

5. A method according to any one of claims 1 to 4, wherein the γ-butyrobetaine hydroxylase is a *Neurospora crassa* γ-butyrobetaine hydroxylase.

6. A method according to any one of claims 1 to 5, wherein the nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH) is a genomic fragment having the nucleotide sequence shown in Figure 7 (SEQ ID NO: 1).

7. A method according to any one of claims 3 to 6, wherein the *Saccharomyces cerevisiae* strain is a FY23 strain.

8. A method according to any one of claims 1 to 7, wherein the non-carnitine synthesising micro-organism is identified by:

applying the micro-organism to a synthetic culture medium containing a non-fermentable carbon source and no carnitine, and which is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain;

culturing the micro-organism; and

detecting no formation of a zone produced in the culture medium in the region of the micro-organism if the micro-organism is not able to produce carnitine.

9. A method according to any one of claims 1 to 8, wherein the success of producing a micro-organism that can biosynthesise carnitine is determined by

applying the transformed micro-organism to a synthetic culture medium containing a non-fermentable carbon source and no carnitine, and which is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain;

culturing the micro-organism; and

detecting the formation of a zone produced in the culture medium in the region of the micro-organism if the micro-organism is able to produce carnitine.

10. A strain of *Saccharomyces cerevisiae* that can produce carnitine in the presence of γ-butyrobetaine, which has been transformed with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH).

11. A *Saccharomyces cerevisiae* strain according to claim 10, wherein the γ-butyrobetaine hydroxylase is a *Neurospora crassa* γ-butyrobetaine hydroxylase.

12. A *Saccharomyces cerevisiae* strain according to either of claims 10 or 11, wherein the nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH) is a genomic fragment having the nucleotide sequence shown in Figure 7 (SEQ ID NO: 1).

13. A *Saccharomyces cerevisiae* strain according to any one of claims 10 to 12, wherein the *Saccharomyces cerevisiae* strain is a FY23 strain.

14. A method of identifying a carnitine-producing micro-organism, the method including the steps of:

applying a micro-organism to a synthetic culture medium which contains a non-fermentable carbon source and no carnitine, and which is coated with a layer of *Saccharomyces cerevisiae* Δ*cit2* strain;

culturing the micro-organism; and

detecting formation of a zone produced in the culture medium in the region of the micro-organism if the micro-organism is a carnitine-producing micro-organism.

15. A method according to claim 14, wherein the culture medium is illuminated or transilluminated in order to detect formation of the zone.

16. A method according to either of claims 14 or 15, wherein the culture medium is agar.

17. A method according to any one of claims 14 to 16, wherein the non-fermentable carbon source is ethanol.

18. A method according to claim 17, wherein the culture medium contains 2% ethanol synthetic agar medium.

19. A method according to any one of claims 14 to 18, wherein the micro-organism is cultured for a period of about 10 days at about 30 °C.

20. A method of producing carnitine, the method including the step of culturing in the presence of γ-butyrobetaine hydroxylase (BBH) a micro-organism that has been transformed with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH).

21. A method according to claim 20, wherein the micro-organism is included in the production of a beverage or food product.

22. A method of enhancing the nutritional value of a beverage or food product, the method including the step of incorporating a micro-organism that has been transformed with a nucleotide sequence encoding γ-butyrobetaine hydroxylase (BBH) in the process of manufacturing the food or beverage product.

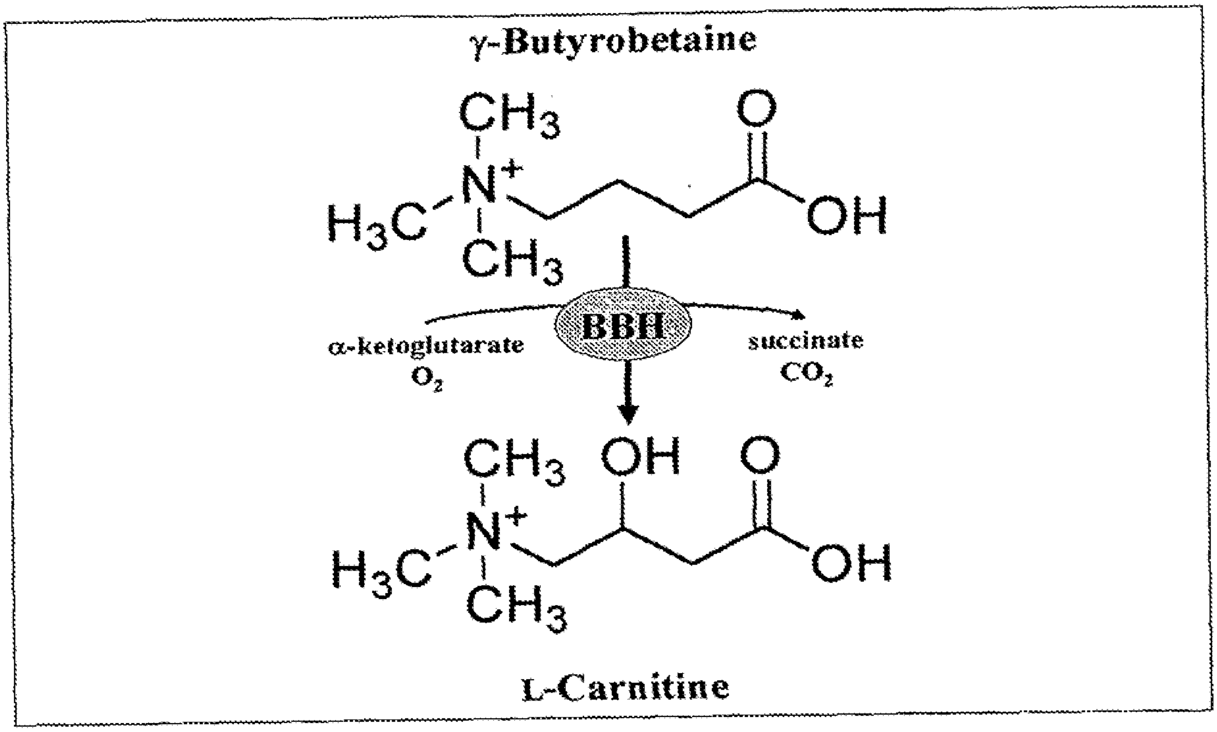
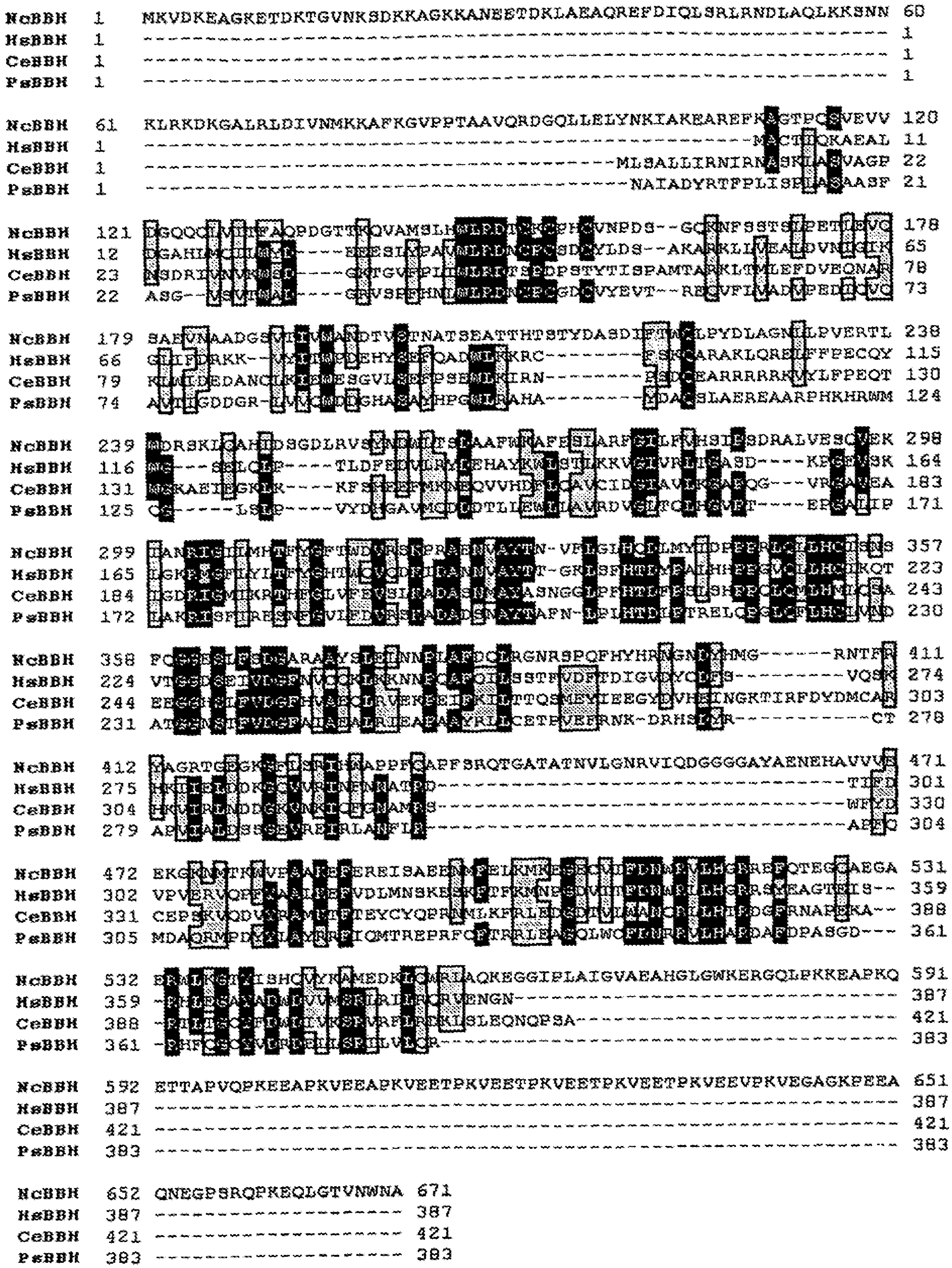

γ-Butyrobetaine
CH3
O
N+
H3C
OH
CH3
BBH
α-ketoglutarate
O2
succinate
CO2
CH3
OH
O
N+
H3C
OH
CH3
L-Carnitine

Figure 1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HsBBH 1 -- 1
CeBBH 1 -- 1
PsBBH 1 -- 1
NcBBH 61 KLRKDKGALRLDIVNMKKAFKGVPPTAAVQRDGQLLELYNKIAKEAREFKAGTPCSVEVV 120
HsBBH 1 11
CeBBH 1 22
PsBBH 1 21
NcBBH 121 178
HsBBH 12 65
CeBBH 23 78
PsBBH 22 73
NcBBH 179 238
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HsBBH 275 301
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PsBBH 279 304
NcBBH 472 531
HsBBH 302 359
CeBBH 331 388
PsBBH 305 361
NcBBH 532 591
HsBBH 359 387
CeBBH 388 421
PsBBH 361 383
NcBBH 592 ETTAPVQPKEEAPKVEEAPKVEETPKVEETPKVEETPKVEETPKVEEVPKVEGAGKPEEA 651
HsBBH 387 -- 387
CeBBH 421 -- 421
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PsBBH 383 -------------------- 383

Figure 2

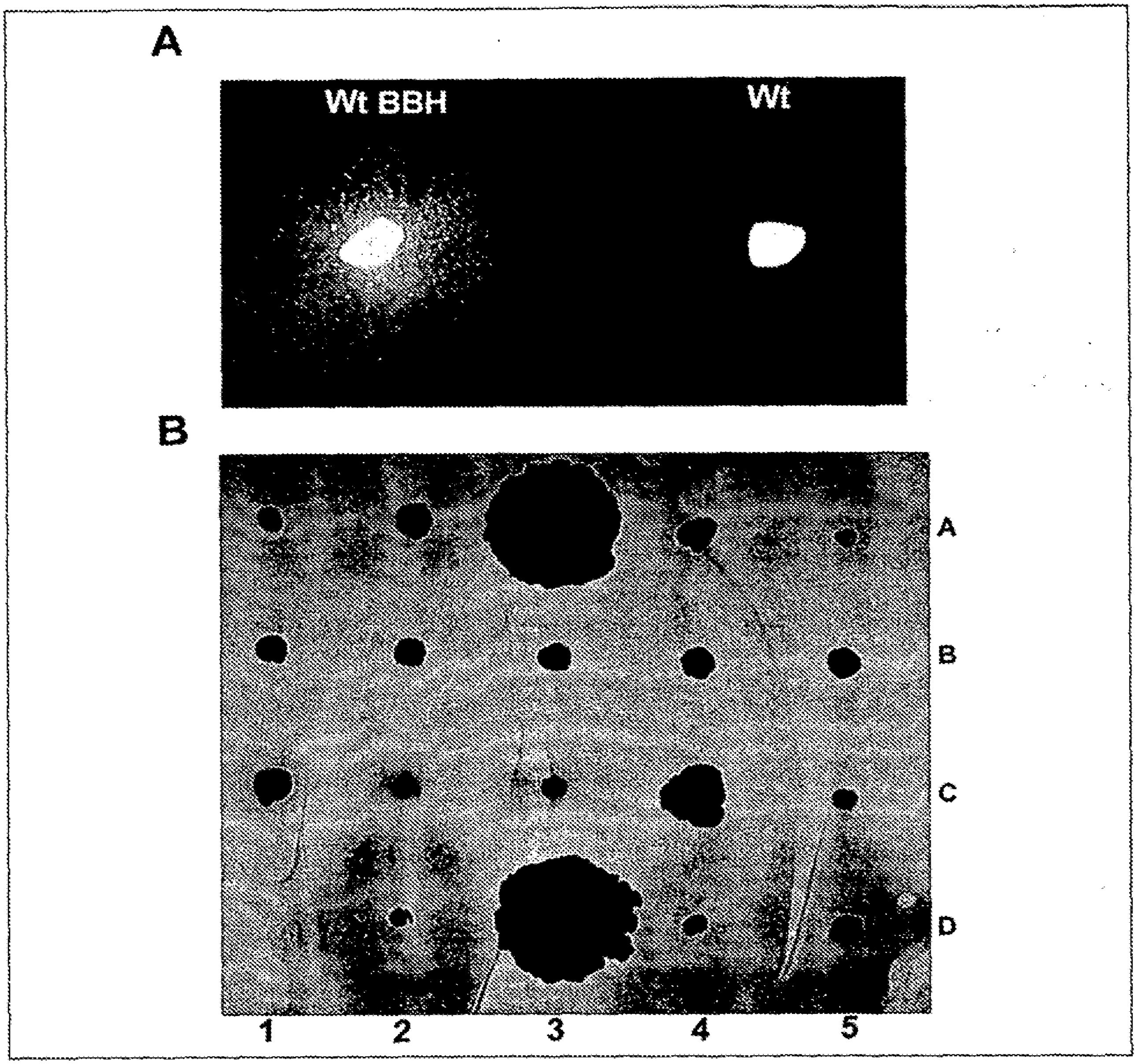
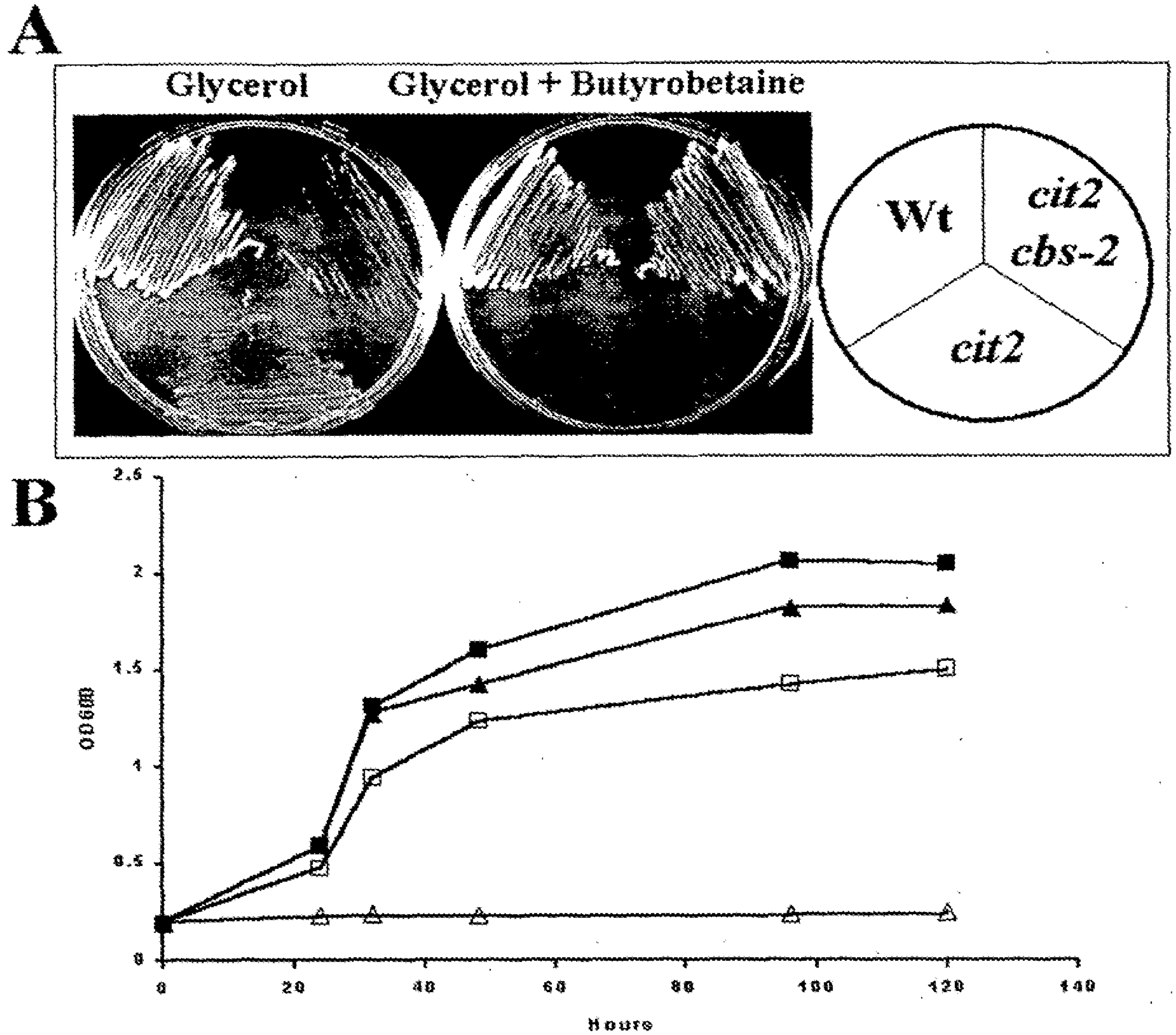


Figure 3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Glycerol + Butyrobetaine
Wt
cit2
cbs-2
cit2
B
OD600
Hours

Figure 4

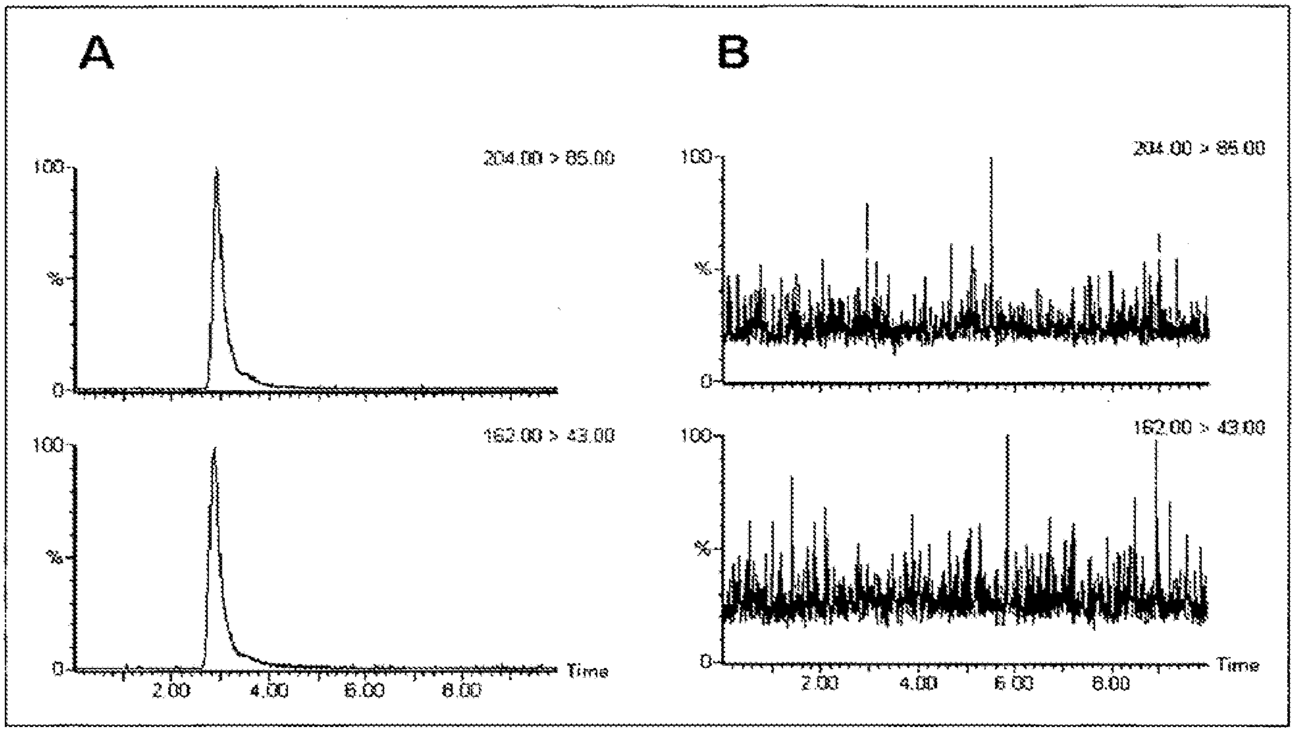

Figure 5

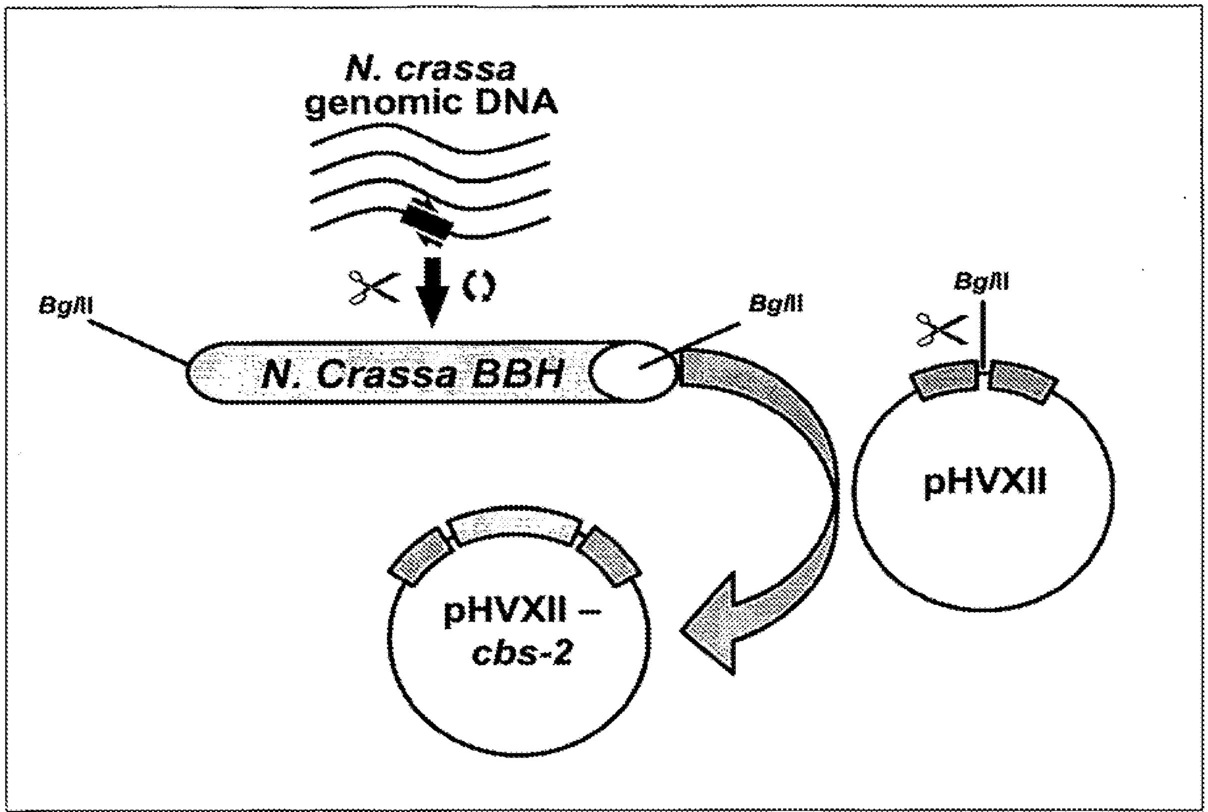
N. crassa
genomic DNA
BglII
BglII
BglII
N. Crassa BBH
pHVXII
pHVXII –
cbs-2

Figure 6

```
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Figure 7

```
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Figure 8

PA137070-PCT as filed.ST25
SEQUENCE LISTING

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<130> PA137070/PCT

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<400> 2

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            20                  25                  30

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        35                  40                  45

Arg Asn Asp Leu Ala Gln Leu Lys Lys Ser Asn Asn Lys Leu Arg Lys
    50                  55                  60

Asp Lys Gly Ala Leu Arg Leu Asp Ile Val Asn Met Lys Lys Ala Phe
65                  70                  75                  80

Lys Gly Val Pro Pro Thr Ala Ala Val Gln Arg Asp Gly Gln Leu Leu
                85                  90                  95

Glu Leu Tyr Asn Lys Ile Ala Lys Glu Ala Arg Glu Phe Lys Ala Gly
            100                 105                 110

Thr Pro Gln Ser Val Glu Val Val Asp Gly Gln Gln Gln Leu Val Ile
        115                 120                 125

Thr Phe Ala Gln Pro Asp Gly Thr Thr Lys Gln Val Ala Met Ser Leu
    130                 135                 140

His Trp Leu Arg Asp Thr Cys Lys Cys Pro His Cys Val Asn Pro Asp
145                 150                 155                 160

Ser Gly Gln Lys Asn Phe Ser Ser Thr Ser Leu Pro Glu Thr Leu Glu
                165                 170                 175

Val Gln Ser Ala Glu Val Asn Ala Ala Asp Gly Ser Val Thr Ile Val
            180                 185                 190

Trp Ala Asn Asp Thr Val Ser Thr Asn Ala Thr Ser Glu Ala Thr Thr
        195                 200                 205
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His Thr Ser Thr Tyr Asp Ala Ser Asp Ile Phe Thr Trp Gln Leu Pro
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Tyr Asn Asp Trp Leu Thr Ser Asp Ala Ala Phe Trp Lys Ala Phe Glu
            260   265   270

Ser Leu Ala Arg Phe Gly Ile Leu Phe Val His Ser Ile Pro Ser Asp
        275   280   285

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2005/003352

A. CLASSIFICATION OF SUBJECT MATTER
C12N1/16 C07K14/37 C12N9/02 C12Q1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAZ F M ET AL: "Carnitine biosynthesis: identification of the cDNA encoding human gamma-butyrobetaine hydroxylase." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. 18 SEP 1998, vol. 250, no. 2, 18 September 1998 (1998-09-18), pages 506-510, XP002365661 ISSN: 0006-291X page 507, column 2, paragraph 2 - page 509, paragraph 2	1-4,10
Y		5-7, 11-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2006	21/03/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Deleu, L

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2005/003352

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GALAGAN JAMES E ET AL: "The genome sequence of the filamentous fungus Neurospora crassa." NATURE. 24 APR 2003, vol. 422, no. 6934, 24 April 2003 (2003-04-24), pages 859-868, XP002365858 ISSN: 0028-0836 the whole document & DATABASE UniProt [Online] 1 March 2004 (2004-03-01), "Hypothetical protein." retrieved from EBI accession no. UNIPROT:Q7S3G2 Database accession no. Q7S3G2 the whole document	5-7, 11-13
Y	LI X ET AL: "Identification of a novel family of nonclassic yeast phosphatidylinositol transfer proteins whose function modulates phospholipase D activity and Sec14p-independent cell growth." MOLECULAR BIOLOGY OF THE CELL. JUN 2000, vol. 11, no. 6, June 2000 (2000-06), pages 1989-2005, XP002365662 ISSN: 1059-1524 figure 5	8,9,14, 15
Y	SWIEGERS J H ET AL: "Carnitine-dependent metabolic activities in Saccharomyces cerevisiae: three carnitine acetyltransferases are essential in a carnitine-dependent strain." YEAST (CHICHESTER, ENGLAND) MAY 2001, vol. 18, no. 7, May 2001 (2001-05), pages 585-595, XP002365859 ISSN: 0749-503X figure 1	8,9,14, 15
X	EP 0 410 430 A (LONZA AG) 30 January 1991 (1991-01-30) page 2, line 29 - line 33; claim 1 page 2, line 38	20
X	PRETORIUS I S ET AL: "Meeting the consumer challenge through genetically customized wine-yeast strains" TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 20, no. 10, 1 October 2002 (2002-10-01), pages 426-432, XP004379928 ISSN: 0167-7799 page 431, column 1, paragraph 2	21,22

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2005/083089 A (CJ CORPORATION; CHUNG, SUNG OH; LEE, BHEONG-UK; KANG, WHAN-KOO; JU, JA) 9 September 2005 (2005-09-09) the whole document	1,2,5
A	VAZ F M ET AL: "Carnitine biosynthesis in mammals" BIOCHEMICAL JOURNAL 01 FEB 2002 UNITED KINGDOM, vol. 361, no. 3, 1 February 2002 (2002-02-01), pages 417-429, XP002370939 ISSN: 0264-6021 figure 3	12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2005/003352

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
EP 0410430	A	30-01-1991	AT	132535	T	15-01-1996
			AU	625525	B2	16-07-1992
			AU	5983790	A	31-01-1991
			BR	9003672	A	27-08-1991
			DD	296702	A5	12-12-1991
			DE	59010027	D1	15-02-1996
			DK	410430	T3	29-01-1996
			ES	2081878	T3	16-03-1996
			FI	102083	B1	15-10-1998
			IE	902689	A1	27-02-1991
			IL	95196	A	26-05-1995
			JP	2018232	C	19-02-1996
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			JP	7051071	B	05-06-1995
			MX	170707	B	08-09-1993
			NO	903338	A	29-01-1991
WO 2005083089	A	09-09-2005	NONE			